Running title: Pressure Effects on the *Shewanella* Chimeric IPMDH

Pressure Effects on the Chimeric 3-Isopropylmalate Dehydrogenases of the Deep-Sea Piezophilic *Shewanella benthica* and the Atmospheric Pressure Adapted *Shewanella oneidensis*

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The chimeric 3-isopropylmalate dehydrogenase enzymes were constructed from the deep-sea piezophilic *Shewanella benthica* and the shallow water *Shewanella oneidensis* genes. The properties of the enzymatic activities under pressure conditions indicated that the central region, which contained the active center and the dimer forming domains, was shown to be the most important region for pressure tolerance in the deep-sea enzyme.

Key words: chimeric enzyme; high pressure; isopropylmalate dehydrogenase; piezophilic bacteria; *Shewanella*

We have isolated a number of piezophilic bacteria from the deep-sea environment in the past twenty years using the manned and unmanned submersibles, Shinkai 6500 and Kaiko systems operated by the Japan Agency for Marine-Earth Science and Technology (JAMSTEC).[1] *Shewanella benthica* DB21MT-2 is one of the isolates from the deepest Mariana Trench Challenger Deep at a depth of 11,000 m and shown to possess an extremely piezophilic growth profile.[2] Enzymatic activities for 3-isopropylmalate dehydrogenase (IPMDH) from *S. benthica* DB21MT-2 were relatively high at higher pressure conditions compared with the IPMDH from *S. oneidensis* MR-1,[3,4] which was isolated from the Oneida Lake at a depth of 6.4 m.[5] Other enzymes, eg. dihydrofolate reductase (DHFR), were also studied relative to the pressure effects on their activities for comparison between the enzymes from deep-sea piezophilic strains and atmospheric adapted strains. Ohmae *et al*. reported that the DHFR of piezophilic *S. violacea* showed

45 enhanced activities up to 100 MPa, and almost the same level of activity at atmospheric
46 pressure (0.1 MPa) remained under 250 MPa conditions. However, the activities of
47 atmospheric adapted *Escherichia coli*'s DHFR were remarkably decreased at the
48 elevated pressure conditions.[6] These observations indicated that the enzymes from
49 piezophilic strains could be more pressure tolerant than the enzymes from atmospheric
50 adapted strains, and they might have special structures for adapting to such
51 high-pressure environments for survival at the bottom of the deep-sea. Structural
52 analyses of the *Shewanella* IPMDHs from *S. benthica* DB21MT-2 (SbMT2_IPMDH)
53 and *S. oneidensis* MR-1 (SoMR1_IPMDH) were also performed and both enzymes
54 were almost identical. However, the deep-sea enzyme (SbMT2_IPMDH) had a
55 somewhat larger internal cavity volume than the SoMR1_IPMDH at ambient
56 atmospheric pressure.[7] To elucidate the molecular mechanisms of pressure adaptation
57 in piezophilic proteins, we selected those *Shewanella* IPMDHs as model enzymes. In
58 this study, we describe the construction of non His-tagged chimeric enzymes from those
59 IPMDHs and their activities under elevated pressure conditions in order to determine
60 which regions are important for pressure adaptation.

61 The *leuB* genes encoding the IPMDH proteins of *S. benthica* DB21MT-2 and *S.*
62 *oneidensis* MR-1 were obtained into the vector pUC19.[3] From the sequence of both
63 *leuB* genes, we identified the common restriction endonuclease sites, *Eco*52I and *Hae*II
64 in the structural gene sequences (Supplemental Fig. 1). Using these restriction enzymes,
65 the chimeric *leuB* genes from SbMT2_*leuB* and SoMR1_*leuB* were constructed using
66 various combinations of the *N*-terminal regions (a.a. 1-65), central regions (a.a. 66-328),

67 and C-terminal regions (a.a. 329-364), as shown in Fig. 1. Totally, 8 different chimeric
68 genes including two originals (SbMT2_*leuB* and SoMR1_*leuB*) were constructed on the
69 vector pUC19. Fig. 1

70 The constructed plasmids were introduced into *E. coli* strain HB101 (*pro*$^-$, *leuB*$^-$) and
71 the *leuB*$^+$ transformants were isolated. The transformans were cultivated in LB medium
72 containing ampicillin (50 μg/mL) and IPTG (isopropyl β-D thiogalactopyranoside, 0.5
73 mM) for 18 hours at 30°C with shaking (210 rpm). The chimeric enzymes from those
74 transformants were partially purified by the procedure as described below;
75 The cultivated cells were collected by centrifugation (8,000 rpm for 30 min. at 4°C),
76 and suspended in 10 mM Tris-HCl pH8.0, 1.0 mM EDTA, 1.0 mM β-mercaptoethanol,
77 1.0 mg/mL lysozyme buffer and disrupted by a repeated freeze thawing and sonication
78 procedure. The supernatants were prepared by centrifugation at 15,000 rpm for 30 min.,
79 and then ammonium sulfate was added to a final concentration of 50% saturation. The
80 supernatants were collected by centrifugation at 15,000 rpm for 30 min. and ammonium
81 sulfate to be a final concentration of 55~65% saturation was added. The resultant
82 precipitates were collected by centrifugation at 15,000 rpm for 30 min. and solubilized
83 with buffer I (50 mM Tris-HCl pH 8.0, 1.0 mM β-mercaptoethanol) containing
84 ammonium sulfate to be a final concentration of 25%. The crude enzymes were loaded
85 onto a Phenyl Sepharose 6 Fast Flow column (GE Healthcare, Uppsala, Sweden)
86 equilibrated with buffer I containing 25% ammonium sulfate. Proteins absorbed were
87 eluted with a linear gradient of 25% to 0% ammonium sulfate. The active enzyme
88 fractions were pooled, concentrated with an Amicon® Ultra 30K device (Millipore Co.,

89 Billerica, MA, USA) and purified using a TSKgel G3000SW$_{XL}$ column (Tosoh Co.,
90 Tokyo, Japan) equilibrated with buffer I. The purity of the recombinant enzymes was
91 verified by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis
92 (Supplemental Fig. 2).

93 IPMDH activity was measured in 50 mM Tris-HCl buffer, pH 7.6, containing 300
94 mM KCl, 0.2 mM $MnCl_2$, 0.8 mM NAD^+, and 0.4 mM 3- isopropylmalate, by recording
95 the production of NADH at 340 nm at 25℃, modified from the standard method.[8)]
96 Approximately one unit (NADH μmol/min) of enzyme was used for the assay, and that
97 activity was defined as 100% relative activity. A high-pressure spectrophotometric
98 system (Syn Co., Kyoto, Japan) combined with a Shimadzu spectrophotometer (Model
99 UV1600-PC, Kyoto, Japan) was used to determine the enzyme activity under pressure
100 conditions (0.1 – 300 MPa), following the procedure suggested by the manufacturer.

101 The partially purified chimeric enzymes were assayed under the elevated pressure
102 conditions, and it was clearly shown that the enzymes including the central region of
103 SbMT2_IPMDH were more active under higher-pressure conditions (dotted lines in Fig.
104 2 indicated as the chimeric enzymes, BBB, OBB, BBO and OBO) compared with the
105 enzymes containing the central region of SoMR1_IPMDH (solid lines in Fig. 2
106 indicated as the chimeric enzymes, OOO, BOO, OOB, BOB). However, the chimeric
107 enzymes, OBO and BBO, were shown higher activities than the original deep-sea
108 enzyme, BBB, at up to 200 MPa conditions, and the chimeric enzyme, BOB, was
109 shown more pressure sensitive than the original shallow water enzyme, OOO. Those
110 results indicated that the central region of SbMT2_IPMDH was particularly important

Fig. 2

111 for activity under high-pressure conditions, compared with N-terminus and C-terminus
112 regions. The central region (a.a. 66-328) included the active site and the dimer forming
113 interfaces,[7,9] thus the structure around the active site or the dimer forming interfaces
114 played an important role in high-pressure habitats. Temperature effects were also
115 measured for those chimeric enzymes. The results indicated that the temperature
116 conditions were not significantly affected in the heat stability and optimal conditions of
117 those enzymes (Supplemental Fig. 3).

118 Our observations concluded that the chimeric enzymes including the central region of
119 the deep-sea SbMT2_IPMDH were particularly affected under high-pressure conditions
120 and more pressure tolerant, but the other regions, N-terminal and C-terminal did not
121 appear to be correlated with their pressure habitats, however C-terminal was less
122 identical than other amino acids sequences between both enzymes (Supplemental Fig.
123 1). The C-terminus regions were expected to be some of the pressure effects according
124 to the predicted 3D structures by the 3D-JIGSAW program (ver. 2.0),[10] but the X-ray
125 crystallography of those enzymes indicated that no significant structural changing was
126 identified[7]. The central regions contained the active sites, Arg97, 107 and 136[9]
127 commonly, and there were identified some difference amino acids around the active
128 sites in the 3D structures, Leu106, Ser266 and Ala268 in SoMR1_IPMDH, and Met106,
129 Ala266 and Ile268 in SbMT2_IPMDH, respectively.[7] We also found some differences
130 around the dimer forming regions, Ara127, Arg128, Val132 and Ser156 in
131 SoMR1_IPMDH, and Glu127, Lys128, Ile132 and Gln156 in SbMT2_IPMDH,
132 respectively (Supplemental Fig. 1). In the case of thermophilic IPMDH, the

hydrophobic interactions at the subunit-subunit interface played important roles in protein stability.[11,12] Therefore, it is possible that the small structural differences in the central regions between SoMR1_ and SbMT2_IPMDHs, caused by the some amino acids differences indicated before, also play significant adaptation roles in extreme pressure habitats. The structural analysis of both enzymes indicated that SbMT2_IPMDH was in a more open form and has a larger cavity volume than SoMR1_IPMDH under atmospheric pressure conditions.[7] This loosely packed structure of the SbMT2_IPMDH might help it to avoid pressure-induced distortion of the native structure and maintain activity at higher pressures. To our knowledge, this is the first observation of the chimeric enzymes from mesophilic and piezophilic enzymes at the elevated pressure conditions. The central regions of this study contain the active site and the dimer forming interfaces, and it is still not clear which is more important for the pressure tolerance. Further experimental studies on the IPMDH pressure adaptation mechanisms involving the 3D-structural analyses under high pressure are now in progress.

Acknowledgments

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References

1) Kato C, "Extremophiles Handbook," eds. Horikoshi K, Antranikian G, Bull A, Robb F and Stetter K, Springer-Verlag, Tokyo, pp. 643-655 (2011).

2) Kato C, Li L, Nogi Y, Nakamura Y, Tamaoka J, and Horikoshi K, *Appl. Environ. Microbiol.,* **64**, 1510-1513 (1998).

3) De Poorter LMI, Suzaki Y, Sato T, Tamegai H, and Kato C, *Mar. Biotechnol.,* **6**, S190-194 (2004).

4) Kasahara R, Sato T, Tamegai H, and Kato C, *Biosci. Biotechnol. Biochem.*, **73**, 2541-2543 (2009).

5) Venkateswaran K, Moser DP, Dollhopf ME, Lies DP, Saffarini DA, MacGregor BJ, Ringelberg DB, White DC, Nishijima M, Sano H, Burghardt J, Stackebrandt E, and Nealson KH, *Int. J. Syst. Bacteriol.,* **49**, 705–724 (1999).

6) Ohmae E, Kubota K, Nakasone K, Kato C, and Gekko K, *Chem. Lett.,* **33**, 798-799 (2004).

7) Nagae T, Kato C, and Watanabe N, *Acta Cryst.*, **F68**, 265-268 (2012).

8) Tamakoshi M, Nakano Y, Kakizawa S, Yamagishi A, and Oshima T, *Extremophiles,* **5**, 17–22 (2001).

9) Nango E, Yamamoto T, Kumasaka T, and Eguchi T, *Bioorganic Med. Chem.,* **17,** 7789-7794 (2009).

10) Kato C, Sato T, Abe F, Ohmae E, Tamegai H, Nakasone K, Siddiqui KS, and Thomas T, "Protein Adaptation in Extremophiles", eds. Siddiqui KS, and Thomas T,

Nova Science Publishers, pp. 167-191 (2008).

11) Kirino H, Aoki M, Aoshima M, Hayashi Y, Ohba M, Yamagishi A, Wakagi T, and Oshima T, *Eur. J. Biochem.*, **220**, 275-281 (1994).

12) Akanuma S, Yamagishi A, Tanaka N, and Oshima T, *Eur. J. Biochem.*, **260**, 499-504 (1999).

Figure legends:

Fig. 1. Structures of the Chimeric *leuB* Genes and their Expressed Protein's ID.

Fig. 2. Effects of Pressure on the Chimeric IPMDH Activities.

Relative activities were measured in the pressurized cells 2 min after the application of pressure. Symbols; □: BBB, ○: BBO, △: OBB, ▽: OBO, ■: OOO, ●: OOB, ▲: BOO, ▼: BOB. Dotted lines: central region is from SbMT2_IPMDH, solid lines: central region is from SoMR1_IPMDH.

Fig. 1

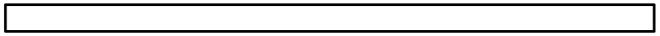
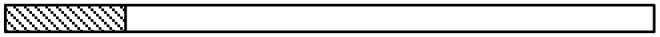
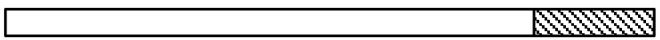
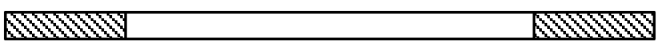
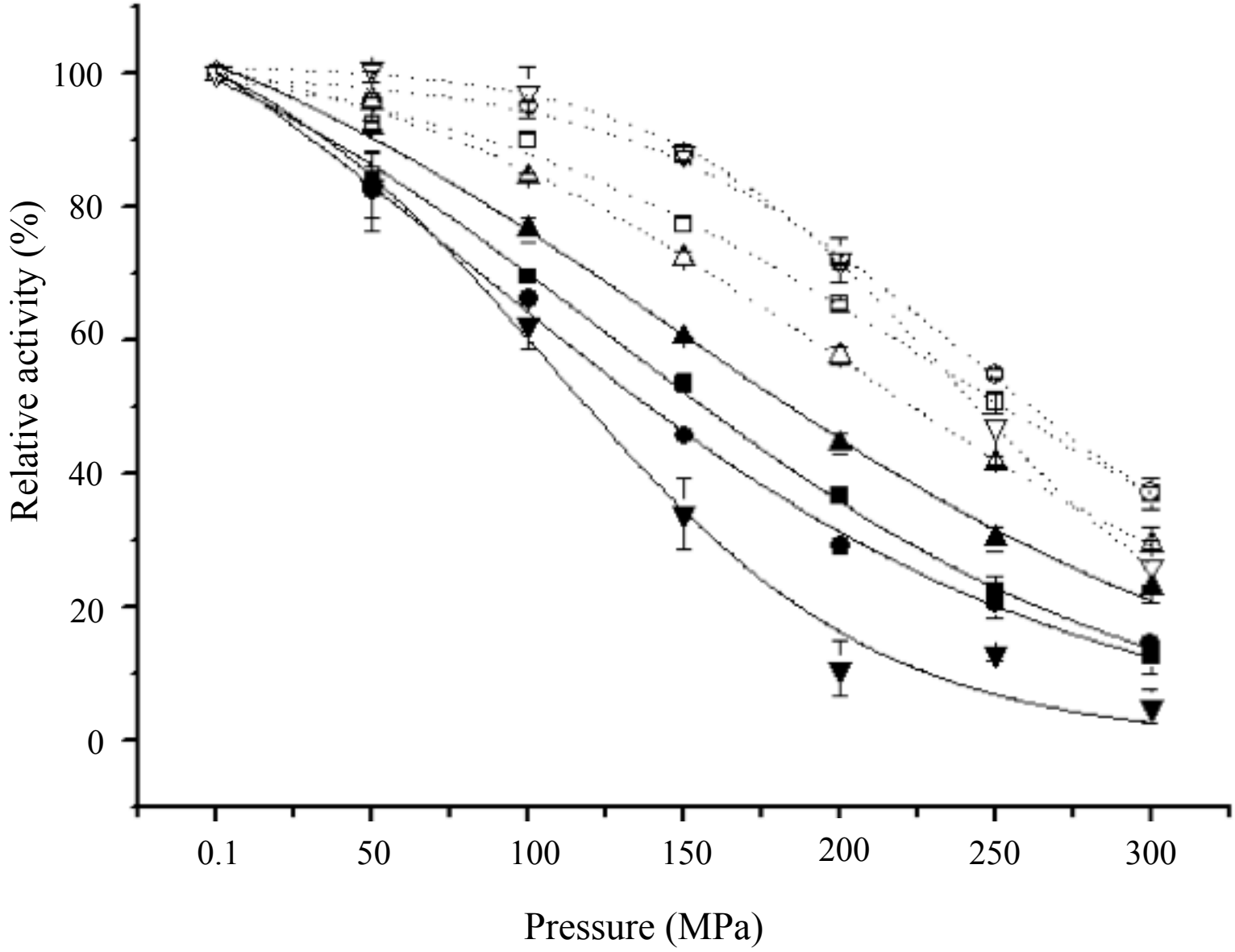
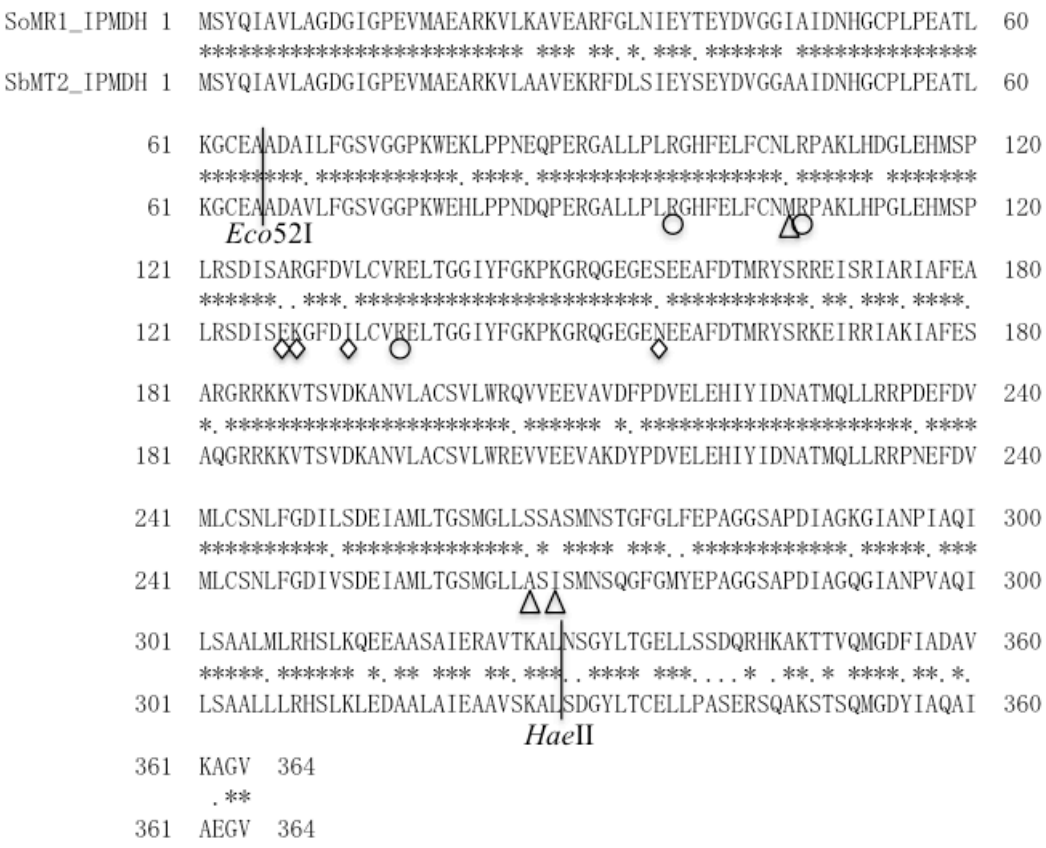
Genes	Chimeric structure	Proteins' ID
SoMR1_*leuB*		OOO
SbMT2_*leuB*		BBB
Sobb_*leuB*		OBB
Sboo_*leuB*		BOO
Soob_*leuB*		OOB
Sbbo_*leuB*		BBO
Sobo_*leuB*		OBO
Sbob_*leuB*		BOB

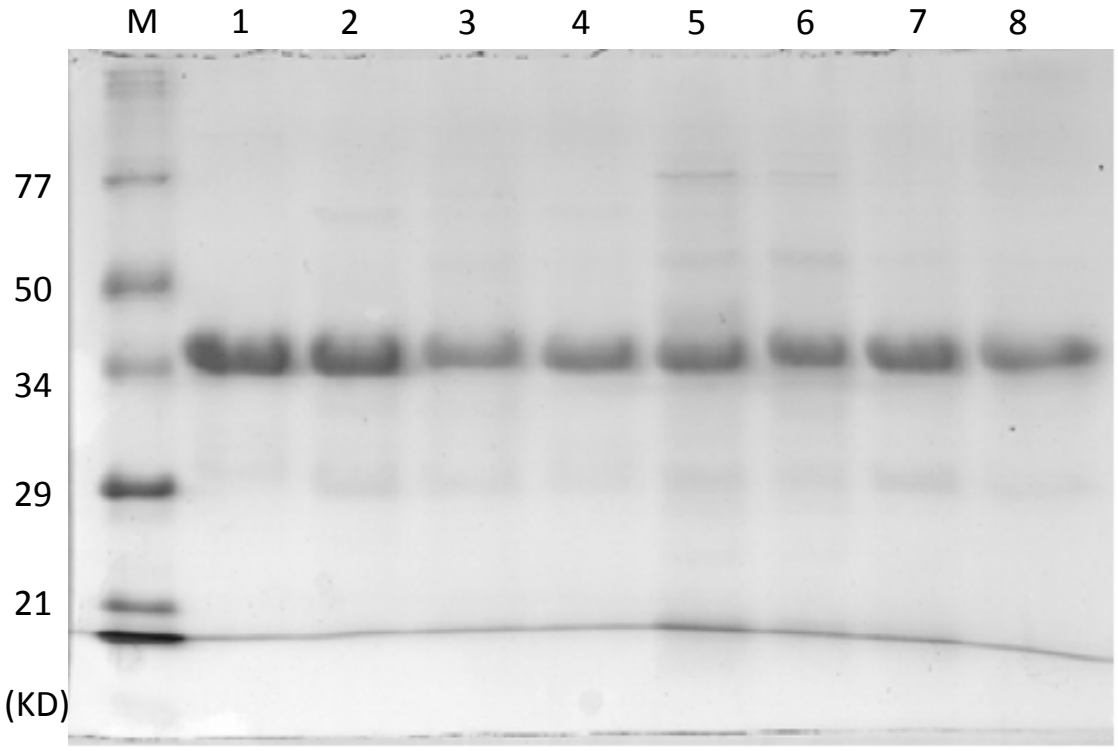
Fig. 2


Relative activity (%)
100
80
60
40
20
0
0.1
50
100
150
200
250
300
Pressure (MPa)



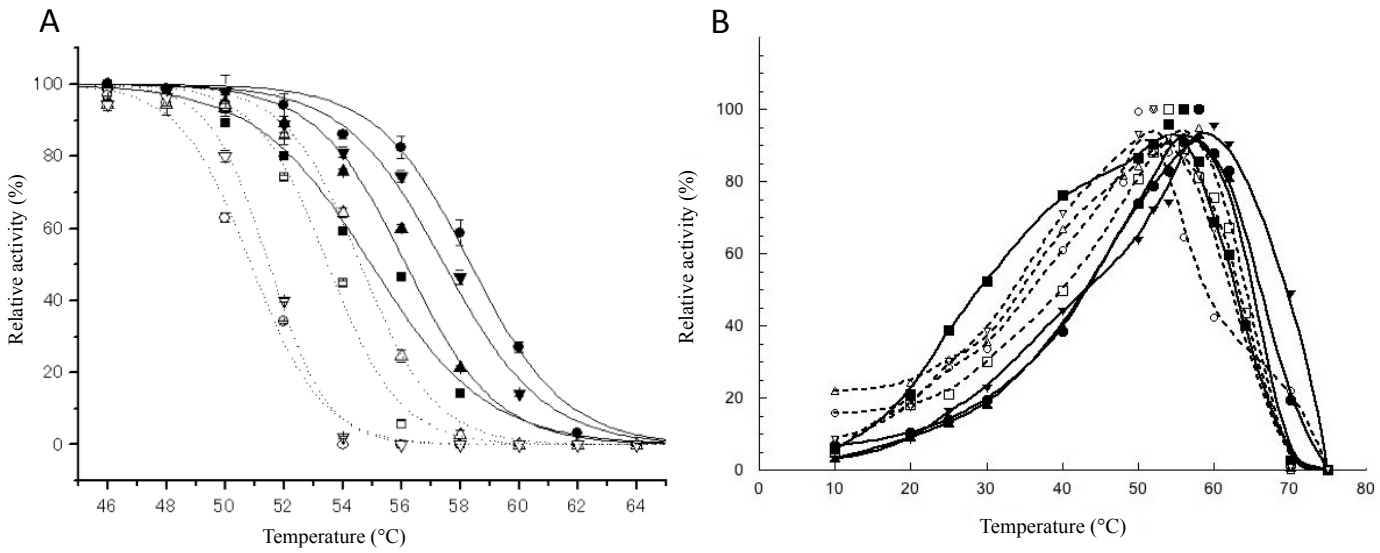
Supplemental Fig. 1. Amino Acid Sequences of the IPMDH Proteins of *S. oneidensis* (SoMR1_IPMDH, AE014299) and *S. benthica* DB21MT-2 (SbMT2_IPMDH, AB506073).

"|" indicates the restriction sites for *Eco*T52I and *Hae*II and "○", "△", and "◇" indicates the active sites, the different amino acids around the active site in 3D structure, and the different amino acids in dimer forming domain, in both enzymes, respectively.



Supplemental Fig. 2. SDS-PAGE Profiles of the Purified Chimeric Enzymes.

Lanes, M: Molecular weight marker, 1: BBB, 2: BBO, 3: OBB, 4: OBO, 5: OOO, 6: OOB, 7: BOO, 8: BOB.



Supplemental Fig. 3. Temperature Effects on the Chimeric Enzymes' Activity.

(A) Temperature stability of the chimeric IPMDHs. (B) Optimal temperatures on the chimeric enzymes' activity. Symbols and lines are the same as in Fig. 2.

Heat stabilities of the purified enzymes were measured by incubating the enzymes with buffer I under various temperature conditions (10 – 70°C) for 30 min. and immediately chilling the enzymes in an ice water bath. The aggregated protein was then removed by centrifugation (15,000 rpm for 5 min), and IPMDH activity of the supernatant was measured. Optimal temperatures for the enzyme activities were also measured under 10 to 70°C conditions, particularly each 2°C increment from 50 to 66°C.